

TITLE OF THE INVENTION

ACETYL AMINO ACID RACEMASE FROM AMYCOLATOPSIS  
ORIENTALIS FOR RACEMIZING CARBAMOYL AMINO ACIDS

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BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to the use of an N-acetyl amino acid racemase (AAR) in a process for the racemization of N-carbamoyl amino acids.

Description of the Background

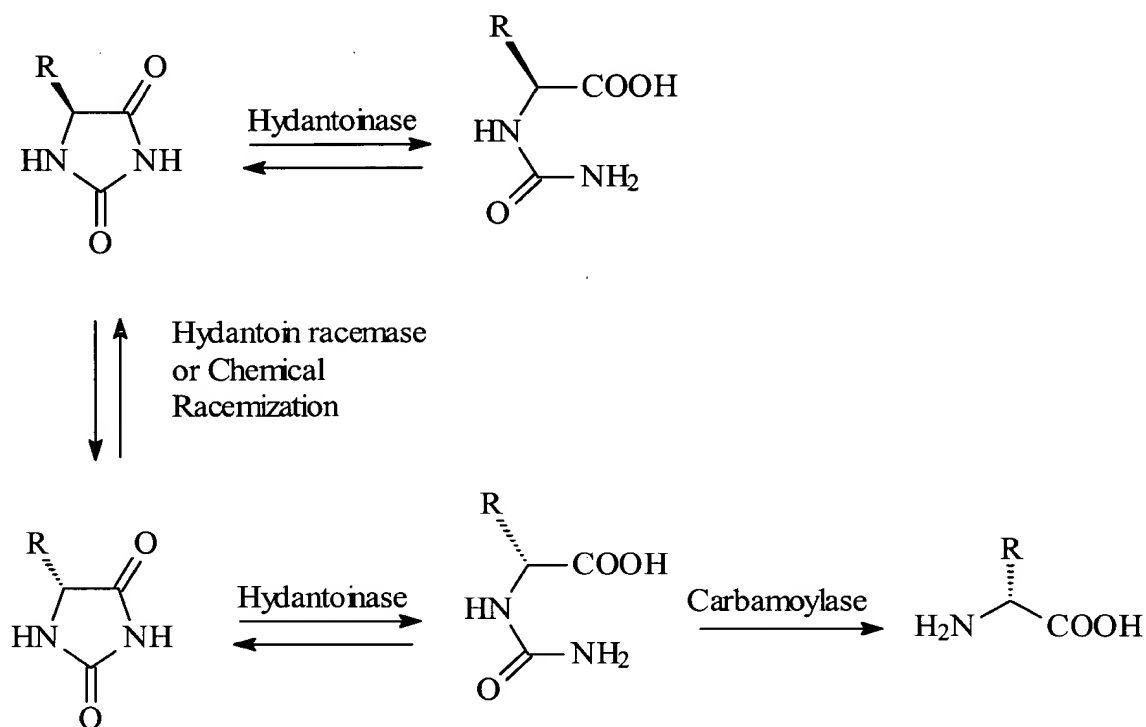
[0002] Optically pure amino acids are important starting materials for chemical synthesis and for parenteral nutrition. Many methods of preparing optically pure amino acids are known. Enzymatic processes, i.a. are suitable in this respect since, on the one hand, they operate catalytically and, on the other hand, permit the preparation of the amino acids with very high enantiomeric enrichment.

[0003] A known enzymatic process starts from racemic hydantoins which are transformed to N-carbamoyl-protected amino acids by means of hydantoinases. These are then converted by carbamoylases to the corresponding amino acids.

[0004] The separation of the racemates occurring in this reaction sequence takes place preferably on the basis of the N-carbamoyl-protected amino acids because both L- and D-selective carbamoylases are available (Park et al., Biotechnol. Prog. 2000, 16, 564-570; May et al., Nat Biotechnol. 2000, 18, 317-20; Pietzsch et al., J. Chromatogr. B Biomed. Sci. Appl. 2000, 737, 179-86; Chao et al., Biotechnol. Prog. 1999, 15, 603-7; Wilms et al., J. Biotechnol. 1999, 68, 101-13; Batisse et al., Appl. Environ. Microbiol. 1997, 63, 763-6; Buson et al., FEMS Microbiol. Lett. 1996, 145, 55-62, each of which is incorporated herein by reference).

[0005] In order to ensure complete conversion of the hydantoins used to optically pure amino acids, the necessary racemization has taken place hitherto on the basis of hydantoins by chemical or enzymatic means (EP 745678; EP 542098; Scheme 1).

Scheme 1:



[0006] N-acetyl amino acid racemases (AARs) from *Streptomyces atratus* Y-53 (Tokuyama et al., Appl. Microbiol. Biotechnol. 1994, 40, 835-840) and *Amycolatopsis* sp. TS-1-60 (Tokuyama et al., Appl. Microbiol. Biotechnol. 1995a, 42, 853-859) and *Amycolatopsis orientalis* sp. *lurida* (DE 19935268) are known. TS-1-60, however, is found to have a very low activity in the case of N-carbamoyl-protected amino acids. Moreover, this enzyme has the disadvantage of a very high metal ion dependence, which appears to be a drawback for the use of this enzyme in an industrial-scale process.

[0007] Accordingly, there remains a need for improved methods of racemizing N-carbamoyl amino acids which overcome the disadvantages described above.

#### SUMMARY OF THE INVENTION

[0008] The object of the present invention was, therefore, to show the use of an N-acetyl amino acid racemase for the improved racemization of N-carbamoyl amino acids compared to known methods. The intention was that this racemase might be used advantageously on an industrial scale in a process for the preparation of optically pure amino acid starting from racemic hydantoins.

[0009] It was another object of the present invention to provide a process for producing enantiomerically enriched amino acids.

[0010] The objects of the present invention, and others, may be accomplished with a method of racemizing N-carbamoyl amino acids, comprising:

[0011] contacting an N-carbamoyl amino acid with an effective amount of an N-acetyl amino acid racemase (AAR) from *Amycolatopsis orientalis* subspecies *lurida*.

[0012] The objects of the present invention may also be accomplished with a method of producing enantiomerically enriched amino acids, comprising:

[0013] contacting an N-carbamoyl amino acid with an effective amount of an N-acetyl amino acid racemase (AAR) from *Amycolatopsis orientalis* subspecies *lurida*, and

[0014] contacting the racemized N-carbamoyl amino acid with a carbamoylase.

[0015] The objects of the present invention may also be accomplished with a method of producing enantiomerically enriched amino acids, comprising:

[0016] contacting an a hydantoin with a hydantoinase to produce the corresponding N-carbamoyl amino acid,

[0017] contacting an N-carbamoyl amino acid with an effective amount of an N-acetyl amino acid racemase (AAR) from *Amycolatopsis orientalis* subspecies *lurida* to produce a racemized N-carbamoyl amino acid, and

[0018] contacting the racemized N-carbamoyl amino acid with a carbamoylase to produce the corresponding amino acid.

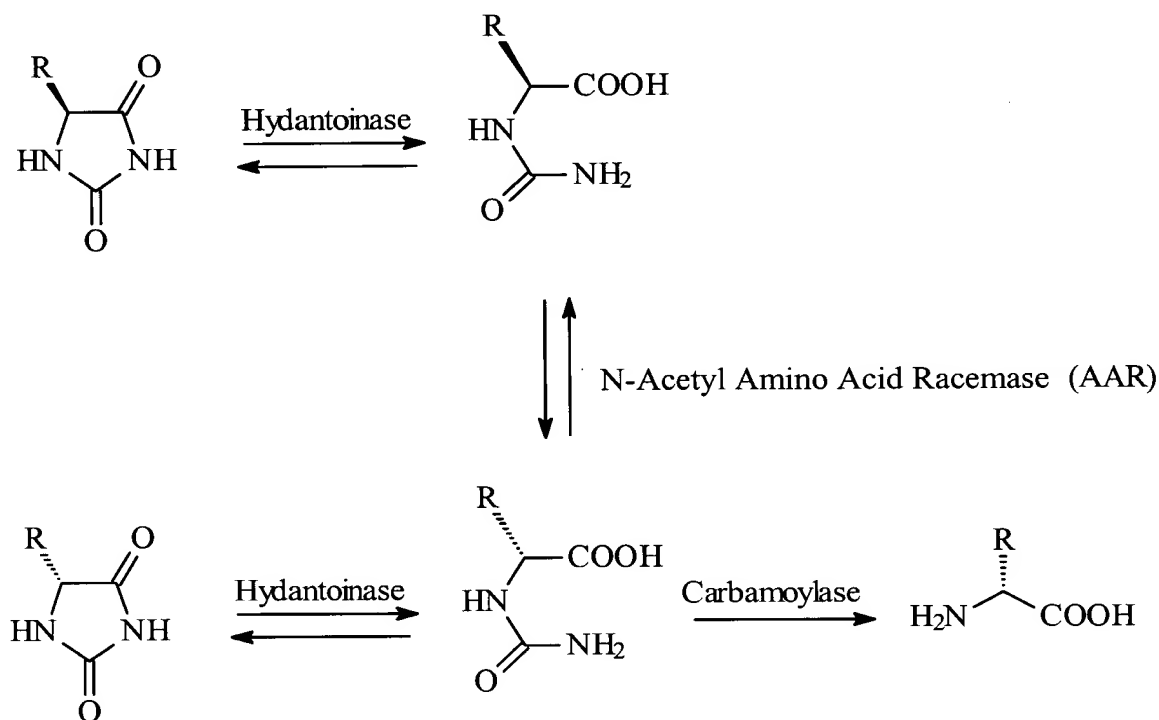
[0019] A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description below.

#### DETAILED DESCRIPTION OF THE INVENTION

[0020] Due to the fact that an N-acetyl amino acid racemase (AAR) from *Amycolatopsis orientalis* subspecies *lurida* (SEQ ID NO.: 2; the encoding nucleic acid sequence is shown in SEQ ID NO.: 1) is used in a process for the racemization of N-carbamoyl amino acids, and in view of the surprisingly high activity of the AAR used according to the invention compared with TS-1-60 in terms of the racemization of N-carbamoyl amino acids, it is possible to achieve an equilibrium of enantiomers of N-carbamoyl-protected amino acids in an improved process.

[0021] This is particularly advantageous in that it is thus possible to establish a further enzymatic step in a process for the preparation of optically pure amino acids which is based on hydantoins (Scheme 2).

Scheme 2:



[0022] In contrast to the enzymatic processes known from the literature and which proceed by way of enzymatic or optionally stressing chemical racemization of hydantoins (Scheme 1), a further advantageous possibility of generating optically pure amino acids from racemic hydantoins has thus been created.

[0023] The variant of AAR from *Amycolatopsis o. sp. lurida* prepared by recombinant technology according to DE 19935268, incorporated herein by reference, is preferably used for the racemization process. It is known from DE 19935268 that this exhibits relatively little heavy metal ion dependence (particularly with regard to cobalt ions) and has low amino acid inhibition. The generation thereof as a recombinant enzyme is also explained therein.

[0024] The process according to the invention, as has been mentioned, is used advantageously in an overall process for the preparation of enantiomerically enriched amino

acids or derivatives thereof starting from hydantoins or N-carbamoyl amino acids. In the case of hydantoins, it is preferable to proceed in such a manner that racemic hydantoins are cleaved by hydantoinases into the corresponding racemic N-carbamoyl amino acids and these are then converted by L- or D-specific carbamoylases into the optically active L- or D-amino acids. To ensure that no enrichment of the unconverted enantiomer of an N-carbamoyl amino acid takes place in the reaction mixture, the enantiomers of the N-carbamoyl amino acids are brought into equilibrium by the addition of the AAR according to the invention and it is thus likewise possible to convert the racemic hydantoin wholly to optically pure amino acids.

[0025] The process of the present invention is preferably conducted in an enzyme-membrane reactor. Such a reactor is described in, for example, DE 199 10 691.6, incorporated herein by reference.

[0026] The enzymes mentioned may be used together or successively in the free form as homogeneously purified compounds or as enzymes prepared by recombinant technology. Moreover, the enzymes may also be used as a constituent of a guest organism (whole-cell catalyst as described in U.S. patent application serial No. 09/407,062, incorporated herein by reference) or in conjunction with the digested cell mass of the host organism. It is also possible to use the enzymes in the immobilized form (Bhavender P. Sharma, Lorraine F. Bailey and Ralph A. Messing, "Immobilisierte Biomaterialien -Techniken and Anwendungen", Angew. Chem. 1982, 94, 836-852, incorporated herein by reference). Immobilization takes place advantageously by freeze-drying (Dordick et al. J. Am. Chem. Soc. 194, 116, 5009-5010, incorporated herein by reference; Okahata et al. Tetrahedron Lett. 1997, 38, 1971-1974, incorporated herein by reference; Adlercreutz et al. Biocatalysis 1992, 6, 291-305, incorporated herein by reference). Freeze-drying in the presence of surfactant substances such as Aerosol OT or polyvinyl pyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol-mono-cetyether) (Goto et al. Biotechnol. Techniques 1997, 11, 375-378, incorporated herein by reference) is more particularly preferred.

[0027] The microorganism *Amycolatopsis orientalis* subsp. *lurida* has been deposited with the German Collection for Microorganisms under the accession number DSM43134.

[0028] The term "AAR" as used herein refers both the native enzyme and the enzyme prepared by recombinant technology.

[0029] The term "enantiomerically enriched" denotes the presence of one enantiomer in the mixture with the other in a proportion of >50%. The proportion, of course, may be higher, such as, for example,  $\geq 60\%$ ,  $\geq 75\%$ ,  $\geq 80\%$ ,  $\geq 90\%$ ,  $\geq 95\%$ ,  $\geq 98\%$ , or  $\geq 99\%$ .

[0030] The term "amino acid" within the context of the present invention means a natural or unnatural  $\alpha$ -amino acid, i.e., the radical situated on the  $\alpha$ -C-atom of the  $\alpha$ -amino acid may be derived from a natural amino acid as described in Beyer-Walter, Lehrbuch der organischen Chemie, S. Hirzel Verlag Stuttgart, 22nd edition, 1991, p.822f., incorporated herein by reference or also from corresponding  $\alpha$ -radicals of unnatural amino acids which are listed, e.g. in DE 19903268.8, incorporated herein by reference.

### EXAMPLES

[0031] Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

#### Detection of Racemase Activity of a Recombinant AAR Enzyme

[0032] The substrate spectrum of the N-acetyl amino acid racemase from *Amycolatopsis orientalis* subsp. *lurida* was tested using the enzyme assay described below.

[0033] The assay was composed of the following:

Tris/HCl buffer	50 mM (pH 8.0)
Substrate	25 mM
Cobalt chloride	6 mM
AAR	approx 150 $\mu$ g purified protein
Final volume	1 ml

[0034] Enantiomerically pure amino acid derivatives were used in the test and the formation of the corresponding racemate was monitored in the polarimeter (Perkin-Elmer 241).

[0035] Incubation took place at 30°C (heated cell) for 3 to 12 hours. The measurements were taken at a wave length of  $\lambda = 365$  nm.

[0036] Table 1: List of the substrates tested and of the corresponding specific activity of the AAR.

Substrate	Specific activity
<i>N</i> -Carbamoyl-D-Met	155 mU/mg
<i>N</i> -Carbamoyl-D-Phe	20 mU/mg
<i>N</i> -Carbamoyl-L-Abs	15 mU/mg
<i>N</i> -Carbamoyl-L-Leu	20 mU/mg
<i>N</i> -Carbamoyl-L-Met	118 mU/mg
<i>N</i> -Carbamoyl-L-Tyr	62 mU/mg
<i>N</i> -Carbamoyl-L-Val	20 mU/mg

[0037] The N-acyl amino acid racemase from A. TS-1-60 with N-carbamoyl-D-Met as substrate has an activity of 100 mU/mg. This specific activity is thus 35% lower than that of the racemase from A. orientalis subsp. lurida.

[0038] Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

[0039] This application is based on German Patent Application serial No. 100 50 124.9, filed on October 11, 2000, which is incorporated herein by reference.